

# Galanin Receptor Subtype GalR2 Mediates Apoptosis in SH-SY5Y Neuroblastoma Cells

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Recently we have shown that galanin binding significantly correlates with survival in neuroblastoma patients, indicating a possible modulatory role of galanin receptors in neuroblastic tumor biology. However, the molecular mechanisms beyond this correlation have not been elucidated. Here, the cellular effects on activation of specific galanin receptor subtypes in human SH-SY5Y neuroblastoma cells were analyzed using a tetracycline-controlled expression system. Pharmacological studies confirmed the inducible expression of high affinity binding sites for galanin in SH-SY5Y cells transfected with the galanin receptors GalR1 (SY5Y/GalR1) and GalR2 (SY5Y/GalR2). Microphysiometry revealed that both receptor subtypes were able to mediate an intracellular signal upon galanin application. Interestingly, induction of receptor expression and treatment with 100 nM galanin resulted in a dramatic decrease in cell viability in SY5Y/GalR2 cells ( $93 \pm 3\%$ )

compared with a less pronounced effect in SY5Y/GalR1 cells ( $19 \pm 10\%$ ). The antiproliferative potency of galanin was 100-fold higher in SY5Y/GalR2 (50% effective concentration, 1.1 nM) than in SY5Y/GalR1 cells (50% effective concentration, 190 nM). Furthermore, activation of receptor expression and exposure to galanin resulted in apparent morphological changes indicative of apoptosis in SY5Y/GalR2 cells only. Induction of cell death by the apoptotic process was confirmed by poly-(ADP-ribose)-polymerase cleavage, caspase-3 activation, and the typical laddering of DNA. This study indicates that a high level of GalR2 expression is able to inhibit cell proliferation and induce apoptosis in neuroblastoma cells and therefore identifies GalR2 as a possible target for pharmacological intervention in neuroblastoma. (*Endocrinology* 145: 500–507, 2004)

THE NEUROPEPTIDE galanin (GAL) is a 29- to 30-amino acid peptide initially isolated from porcine intestine (1). Consistent with its widespread distribution throughout the central and peripheral nervous systems, it has a wide range of effects, including neuromodulatory, reproductive, and endocrine functions (2–4). The actions of GAL are mediated through interaction with at least three specific receptor subtypes that are members of the G protein-coupled receptor superfamily, namely GalR1, GalR2, and GalR3 (5). These receptors have been shown to activate a variety of intracellular second messenger pathways that account for the different physiological effects of GAL (4, 6). The recently identified galanin-like peptide (GALP), which shares 13 identical amino acids with GAL, is an endogenous ligand for the GalR2 receptor (7).

GAL and its receptors have been shown to be expressed in tumors, such as pituitary tumors (8–10) and glioblastomas (11). In addition, GAL as well as its receptors are expressed in neuroblastic tumors, and high levels of GAL binding sites are associated with favorable prognosis (12). A study by Perel *et al.* (13) suggests that GAL influences neuroblastoma

development and tumor differentiation as an autocrine/paracrine modulator.

Neuroblastic tumors, including neuroblastomas and ganglioneuromas, are tumors of the sympathetic nervous system. They arise from neural crest precursors within sympathetic ganglia and the adrenal medulla and represent the most common pediatric extracranial malignancies. These embryonal tumors show a variety of differentiation processes such as complete maturation to a benign ganglioneuroma. Also, spontaneous regression of neuroblastomas occurs at an unusually high rate. The mechanisms underlying the regression are not clear, but a phenomenon similar to apoptosis might be involved (14–16).

As recent findings implicate an involvement of GAL and its receptors in neuroblastic tumor development (12, 13), we further investigated whether GAL is able to influence the proliferation and viability of neuroblastoma cells. The human GAL receptors, GalR1, GalR2, and GalR3, were expressed under the control of a tetracycline-regulated expression system (T-REx System) in the human SH-SY5Y neuroblastoma cell line, which is frequently used as a model system for neuroblastoma growth and differentiation (17).

## Materials and Methods

### Materials

Oligonucleotides used in this study were custom-synthesized by MWG (Ebersberg, Germany). The T-REx System (Invitrogen, Groningen, The Netherlands), a tetracycline-regulated expression system for mammalian cells, was used for GAL receptor expression in the SH-SY5Y cell line. [ $^{125}$ I]GAL (2000 Ci/mmol) was purchased from Amersham Pharmacia Biotech (Little Chalfont, UK), GAL peptide was ob-

Abbreviations: DEVD, Ac-DEVD-CHO; ECAR, extracellular acidification rate; GAL, galanin; GalR1, galanin receptor 1; GalR2, galanin receptor 2; GalR3, galanin receptor 3; GALP, galanin-like peptide; SY5Y/GalR1, SH-SY5Y cells transfected with GalR1; SY5Y/GalR2, SH-SY5Y cells transfected with GalR2; SY5Y/GalR3, SH-SY5Y cells transfected with GalR3; PARP, poly-(ADP-ribose)-polymerase.

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tained from Bachem (Bubendorf, Switzerland), GALP was purchased from Phoenix Pharmaceuticals (Belmont, CA), and AR-M1896 was synthesized by Neosystems (Strasbourg, France).

### Plasmid construction

The cDNAs encoding the human GAL receptors were generated by RT-PCR with total RNA isolated from human cells expressing the respective receptors. 5' and 3' PCR primers were designed based on published human cDNA sequences (18–20). The full-length human GAL receptor cDNAs were subcloned into the expression vector pcDNA4/TO (T-REx System) and confirmed by DNA sequencing.

### Cell culture and transfections

SH-SY5Y neuroblastoma cells (provided by Dr. John Inge Johnson, Tromsø, Norway) were grown in a 1:1 mixture of MEM with Earl's salt and F-12 medium (Sigma-Aldrich Corp., St. Louis, MO) supplemented with 10% fetal calf serum, 200 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml fungizone (BioWhittaker, Walkersville, CA).

To obtain stable cell lines that express GAL receptors upon induction with tetracycline, two consecutive transfections with Lipofectamine Plus reagent (Life Technologies, Inc., Gaithersburg, MD) were performed. SH-SY5Y cells were first transfected with the vector encoding the tetracycline repressor, pcDNA6/TR (T-REx System). Stable transfectants were selected in the presence of 5 µg/ml blasticidin and transferred to 96-well plates. After cells had expanded, *lacZ* reporter gene assays were performed to detect clones expressing the highest levels of tetracycline repressor to ensure the most complete repression of basal transcription of GAL receptors. After transfection with pcDNA4/TO containing the GAL receptors, cells were kept in growth medium containing 5 µg/ml blasticidin as well as 100 µg/ml zeocin for double selection. Single clones were isolated by limiting dilution, transferred into 96-well plates, expanded, and selected by [<sup>125</sup>I]GAL binding assays for the expression of GAL receptors.

### RIA

Cell pellets were treated with 10 vol boiling 1 M acetic acid for 10 min. After addition of trifluoroacetic acid to a final concentration of 1% (vol/vol), the suspension was centrifuged at 13,000 rpm for 10 min at 4 C. Cell culture supernatants were acidified with trifluoroacetic acid (1%, vol/vol). The supernatants were applied to C<sub>18</sub> cartridges (Sep-Pak, Waters Corp., Milford, MA). Peptide elution and RIAs were performed as described by Berger *et al.* (12).

### Membrane preparations and radioligand binding assays

Membranes from transfected SH-SY5Y cells induced overnight with 1 µg/ml tetracycline were prepared as described previously (19). Binding of radiolabeled GAL to membrane preparations (15 µg) was carried out as described by Berger *et al.* (12).

For whole cell binding assays cells were scraped off the tissue flask in 1× PBS with 2.5 mM EDTA and centrifuged at 1000 rpm for 5 min at 4 C. Cells were resuspended in binding buffer [20 mM HEPES (pH 7.5), 5 mM MgCl<sub>2</sub>, 100 mM NaCl, 1 mM EDTA, 10 µg/ml leupeptin, 100 µg/ml bacitracin, and 0.5% (wt/vol) BSA] containing 50 pM [<sup>125</sup>I]-labeled human GAL and incubated for 1 h at room temperature. For the determination of nonspecific binding, 1 µM unlabeled GAL was added. Reactions were terminated by filtration over glass-fiber filters that had been presoaked in 0.3% polyethylenimine (vol/vol). Filters were washed four times with 0.4 ml cold wash buffer [1× PBS (pH 7.0) and 0.01% Triton X-100], dried, and counted in a γ-counter.

Displacement of [<sup>125</sup>I]GAL binding was performed in duplicate in a volume of 120 µl binding buffer containing 15 µg membrane protein, 50 pM [<sup>125</sup>I]GAL, and different concentrations of GAL, GALP, and the GalR2-selective analog AR-M1896 (21). Equilibrium binding assays were performed in duplicate with 15 µg membrane protein in a volume of 120 µl binding buffer containing 0.01–200 nM [<sup>125</sup>I]GAL. Unlabeled GAL (1 µM) was used to determine nonspecific binding for each concentration of [<sup>125</sup>I]GAL. The results were analyzed using PRISM 3.03 software (GraphPad, Inc., San Diego, CA).

### Measurement of extracellular acidification rate

Approximately 3 × 10<sup>5</sup> cells/ml were seeded into 3.0-µm pore size Transwells (Corning Costar, Cambridge, MA) held in 12-well plates. The cells were allowed to settle in normal growth medium for about 7 h and were cultured overnight in serum-free growth medium supplemented with or without 1 µg/ml tetracycline for GAL receptor induction. The extracellular acidification rate of the cells was determined using a cytosensor microphysiometer (Molecular Devices, Ismaning, Germany) as reported previously (6). Curve fitting was carried out using PRISM 3.03 software (GraphPad, Inc.).

### Cell proliferation studies

Cells were seeded into 96-well plates in complete growth medium and were allowed to settle overnight. The next day, cells were induced with 1 µg/ml tetracycline, and induced as well as uninduced cells were treated for 72 h with various concentrations of GAL, GALP, and AR-M1896 or with 50 µM Ac-DEVD-CHO (DEVD; Biomol, Plymouth Meeting, PA) 2 h before tetracycline induction. Due to the short half-life of the added substances, the medium was freshly supplemented with the peptides and changed daily. The number of viable cells was determined using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega Corp., Madison, WI) according to the manufacturer's protocol.

### Preparation of cell lysates and poly-(ADP-ribose)-polymerase (PARP) Western blot analysis

SY5Y/mock, SY5Y/GalR1, and SY5Y/GalR2 cells were treated with 1 µg/ml tetracycline and 100 nM GAL for 48 h. Detached SY5Y/GalR2 cells were collected by centrifugation at 4 C at 1500 rpm for 5 min; adherent cells were scraped off in 1× PBS and centrifuged. PARP Western blot analysis was carried out according to the manufacturer's instructions (Roche, Mannheim, Germany).

### Detection of key caspase-3 activity

SY5Y/mock, SY5Y/GalR1, and SY5Y/GalR2 cells were treated with 1 µg/ml tetracycline and 100 nM GAL or with 3 µM rotenone for 48 h. Caspase-3 activity was assayed by using the BD ApoAlert Caspase Colorimetric Assay Kit according to the instructions provided by the manufacturer (BD Biosciences Clontech, Erembodegem, Belgium).

### DNA fragmentation analysis

To detect DNA ladders due to apoptotic processes, genomic DNA was isolated according to the method of Herget *et al.* (22). Briefly, detached and adherent cells were collected by centrifugation, and the cell pellet was resuspended in 20 µl cell lysis buffer (0.5% Triton X-100, 20 mM EDTA, and 5 mM Tris-HCl, pH 8.0) and incubated with 0.5 mg/ml proteinase K and 0.5 mg/ml ribonuclease A at 50 C for 1 h each. The reactions were stopped by heat treatment at 70 C for 10 min. Fifteen microliters of preheated sample buffer (1% low melting agarose, 10 mM EDTA, 0.25% bromophenol blue, and 40% sucrose) were added, and the samples were loaded onto a 1.4% agarose gel. The DNA ladders were visualized under UV light with ethidium bromide.

### Statistical analysis

Results are expressed as the mean ± SE. Comparisons were performed using Wilcoxon's test. *P* < 0.05 was considered statistically significant.

## Results

### Expression of GAL and GAL receptors in neuroblastoma cell lines

To investigate the effects of GAL and its receptors in neuroblastomas, we screened several neuroblastoma cell lines for endogenous expression of GAL receptors. No cell line was found to express functional GAL receptors (data not shown). However, all cell lines investigated showed expres-

sion and secretion of GAL peptide. The amount of GAL peptide in SH-SY5Y neuroblastoma cells was 37 fmol/liter  $\times$   $10^6$  cells, and the peptide was secreted into the growth medium at a rate of 6.2 fmol/liter  $\times$   $10^6$  cells/h as determined by RIA. To study the role of GAL in these cells we decided to transfect them with the three human GAL receptors known to date. To avoid permanent activation of GAL receptors in SH-SY5Y cells by endogenous GAL peptide, we generated stable transfectants in which GAL receptor expression is under the control of a tetracycline-regulated expression system (T-REx System). Receptor expression was induced overnight with 1  $\mu$ g/ml tetracycline. [ $^{125}$ I]GAL membrane binding studies revealed high binding levels in SY5Y/GalR1 and SY5Y/GalR2, very low GAL binding levels in SY5Y/GalR3, and no binding in SY5Y/mock cells (Table 1). The leakage of receptor expression in uninduced cells was 4% for SY5Y/GalR1 and 2% for SY5Y/GalR2 cells (Table 1). Due to the very low expression of GAL binding sites in SY5Y/GalR3 cells, no further pharmacological analysis of this cell line is presented. Whole cell binding assays confirmed that the observed GAL binding on isolated membranes of the transfected tetracycline-induced cells is due to receptors located on the cell surface. The GAL peptide concentration, measured by RIA, was not significantly altered by transfection of the cells and tetracycline treatment of SY5Y/mock, GalR1, or GalR2 cells (Table 1).

#### Pharmacological profile of the expressed GAL receptors

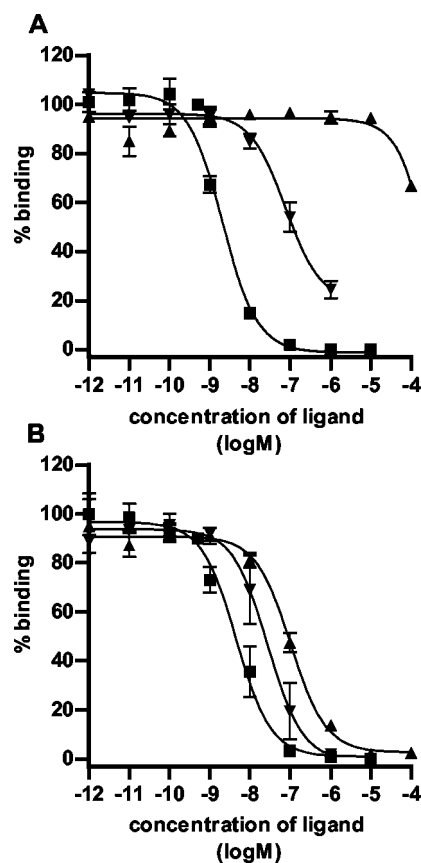
The level of receptor expression in the tetracycline-induced cell lines was determined by saturation binding of [ $^{125}$ I]GAL to membrane preparations. Nonlinear regression analysis of the specific binding data yielded a  $K_d$  of  $11.9 \pm 1.5$  nM and a binding capacity of  $34.6 \pm 1.1$  pmol/mg membrane protein for SY5Y/GalR1 and  $18.5 \pm 2.3$  nM and  $84.1 \pm 2.8$  pmol/mg membrane protein for SY5Y/GalR2 (data are the mean  $\pm$  SE of a representative experiment performed in duplicate;  $n = 2$ ).

To compare the pharmacological properties of GalR1 and GalR2, competition studies with GAL, GALP, and the GalR2-specific analog AR-M1896 (21) were performed (Fig. 1). The affinities of the two receptors for GAL as well as for GALP were in a similar range, whereas the  $IC_{50}$  of GalR1 for AR-M1896 was higher than that for GalR2 (Fig. 1).

**TABLE 1.** GAL binding and GAL peptide levels of transfected SH-SY5Y cells

	pmol bound galanin/mg membrane protein (n = 3)	fmol galanin/ $1 \times 10^6$ cells (n = 2)
SY5Y/mock	BD	31
SY5Y/mock + tet	BD	42
SY5Y/GalR1	$0.4 \pm 0.1$	42
SY5Y/GalR1 + tet	$10.2 \pm 1.2$	45
SY5Y/GalR2	$0.05 \pm 0.016$	40
SY5Y/GalR2 + tet	$3.2 \pm 0.3$	69
SY5Y/GalR3	BD	ND
SY5Y/GalR3 + tet	$0.02 \pm 0.01$	43

BD, Below detection limit ( $\leq 0.005$  pmol bound galanin/mg membrane protein); ND, not done; + tet, treatment of cells with 1  $\mu$ g/ml tetracycline for 12 h.



**FIG. 1.** Pharmacological profile of GAL receptors expressed in tetracycline-induced SH-SY5Y cells. Competition of [ $^{125}$ I]GAL binding by increasing concentrations of unlabeled GAL (■), GALP (▼), and AR-M1896 (▲) in membranes of SH-SY5Y cells transfected with GalR1 (A;  $IC_{50}$  values: GAL,  $2.1 \pm 0.4$  nM; GALP,  $77 \pm 20$  nM; AR-M1896, more than 1000 nM) and with GalR2 (B;  $IC_{50}$  values: GAL,  $4.6 \pm 1.1$  nM; GALP,  $28 \pm 10$  nM; AR-M1896,  $110 \pm 10$  nM). Data are the mean  $\pm$  SE of at least two independent experiments performed in duplicate.

#### Functionality of GAL receptors as determined by cytosensor studies

The functional characteristics of GalR1, GalR2, and GalR3 expression in SH-SY5Y cells were investigated using a microphysiometer that detects small metabolic changes in living cells in real-time as variations in pH in the extracellular environment measured as the extracellular acidification rate (ECAR). GAL application to tetracycline-induced SY5Y/GalR1 and SY5Y/GalR2 cells caused an increase in the ECAR, reaching a plateau after approximately 20 min, whereas tetracycline-induced SY5Y/GalR3 and SY5Y/mock cells were unaffected (Fig. 2). Upon removal of GAL from the superfusion medium, the ECAR gradually decreased and reached basal values after approximately 30 min. The observed increase in ECAR was dependent on the GAL concentration, with an 50% effective concentration ( $EC_{50}$ ) of  $4.9 \pm 1.4$  nM in SY5Y/GalR1 and  $1.2 \pm 0.4$  nM in SY5Y/GalR2 cells. The GalR2-specific analog AR-M1896 altered the ECAR only of SY5Y/GalR2 ( $EC_{50}$ ,  $3.7 \pm 0.9$  nM), not of SY5Y/GalR1 cells (Fig. 2, F and H). In contrast to SY5Y/GalR1 cells, in which three consecutive applications of human GAL showed a similar magnitude of the response, repeated GAL admin-

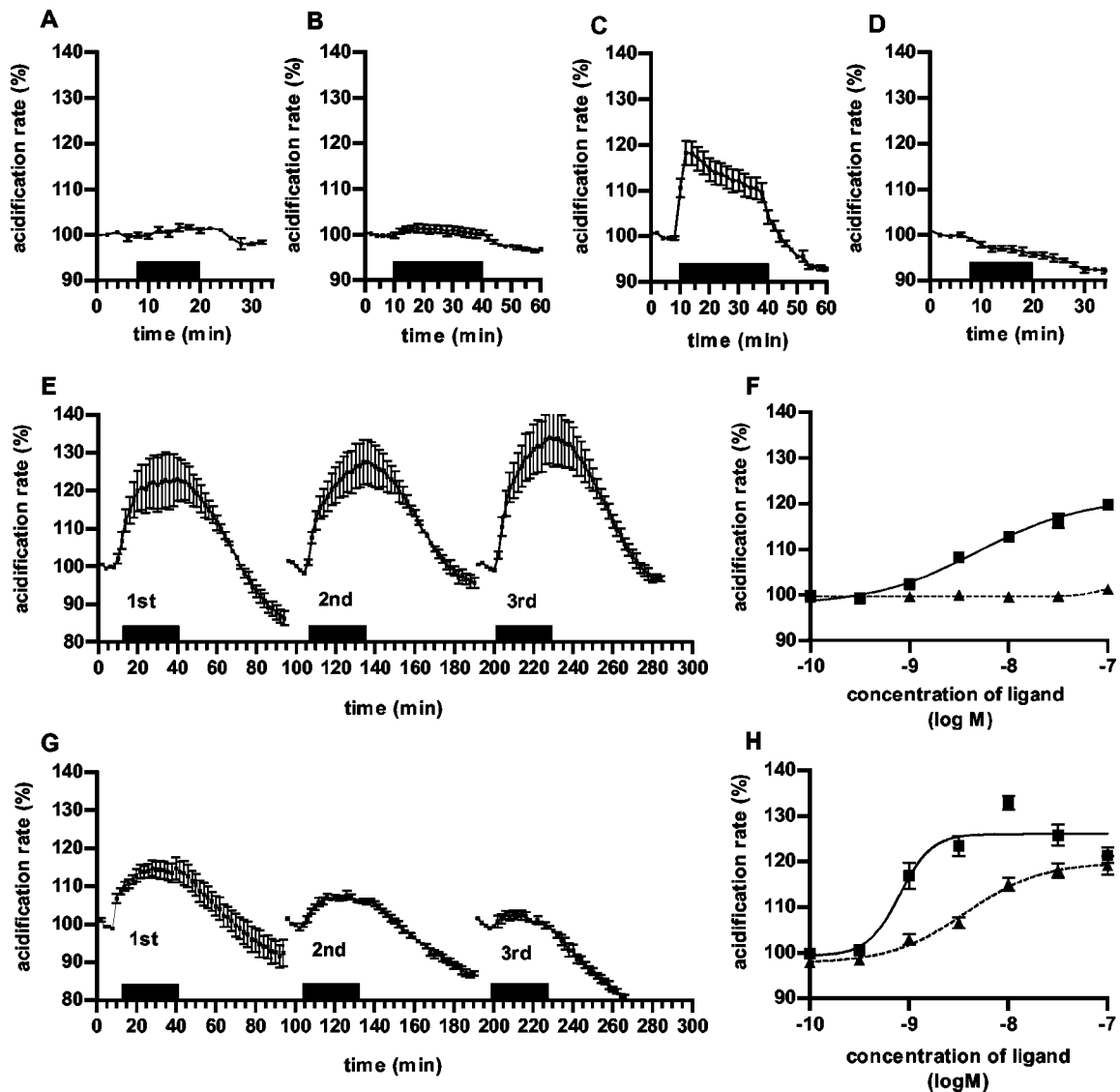


FIG. 2. Metabolic response upon GAL receptor activation in SH-SY5Y cells. Treatment of uninduced SY5Y/mock (A), SY5Y/GalR1 (B), and SY5Y/GalR2 (C) cells with 100 nM GAL. Repeated stimulation with 100 nM GAL in tetracycline-treated SY5Y/GalR1 cells causes similar ECAR amplitudes (E), but desensitization of ECAR responses in SY5Y/GalR2 cells (G) and no changes of ECAR in SY5Y/GalR3 cells (D). Values are the mean  $\pm$  SE ( $n = 3$ ) from a single representative experiment. Dose-response curves for human GAL ( $\blacksquare$ ) and AR-M1896 ( $\blacktriangle$ ) in SY5Y/GalR1 (F) and SY5Y/GalR2 cells (H) are shown. Data are the mean  $\pm$  SE of three independent experiments performed at least in duplicate.

istration resulted in a desensitization of the response of SY5Y/GalR2 cells (Fig. 2, E and G). The leakage of the inducible system, leading to low levels of GAL-binding sites in uninduced SY5Y/GalR1 cells (4%), did not result in a detectable response using microphysiometry (Fig. 2B). In contrast, low levels of expression of GAL receptors in uninduced SY5Y/GalR2 cells (2%) did produce a response of the ECAR to GAL administration ( $EC_{50}$ ,  $28 \pm 1.6$  nM). The magnitude of the ECAR of uninduced SY5Y/GalR2 cells was comparable to that of tetracycline-induced cells (Fig. 2, C and G). However, no desensitization was observed in uninduced SY5Y/GalR2 cells upon repeated GAL treatment.

#### Changes in cell morphology

Uninduced SY5Y/GalR1 and SY5Y/GalR2 cells as well as tetracycline-induced SY5Y/mock and SY5Y/GalR1 cells did

not show morphological changes in response to GAL treatment (Fig. 3). In contrast, morphological examination of GalR2-transfected cells grown in the presence of 1  $\mu$ g/ml tetracycline and 100 nM GAL revealed a dramatic change in cell morphology (Fig. 3). Within 24 h after addition of tetracycline and GAL, cells started to round up and exhibited decreased adhesion to the tissue flask. After 48 h of induction, 60–80% of cells had detached from the culture flask and emerged into the growth medium (Fig. 3O). Addition of 100 nM GALP and 1  $\mu$ M AR-M1896 to induced SY5Y/GalR2 cells caused a similar response (data not shown). Treatment of SY5Y/GalR2 cells with tetracycline alone also showed some morphological alterations, but cells did not detach from the culture flask (Fig. 3M). Pretreatment with the caspase-3 inhibitor DEVD did not alter the response of SY5Y/GalR2 cells to tetracycline and GAL (Fig. 3, N and P). However, stau-

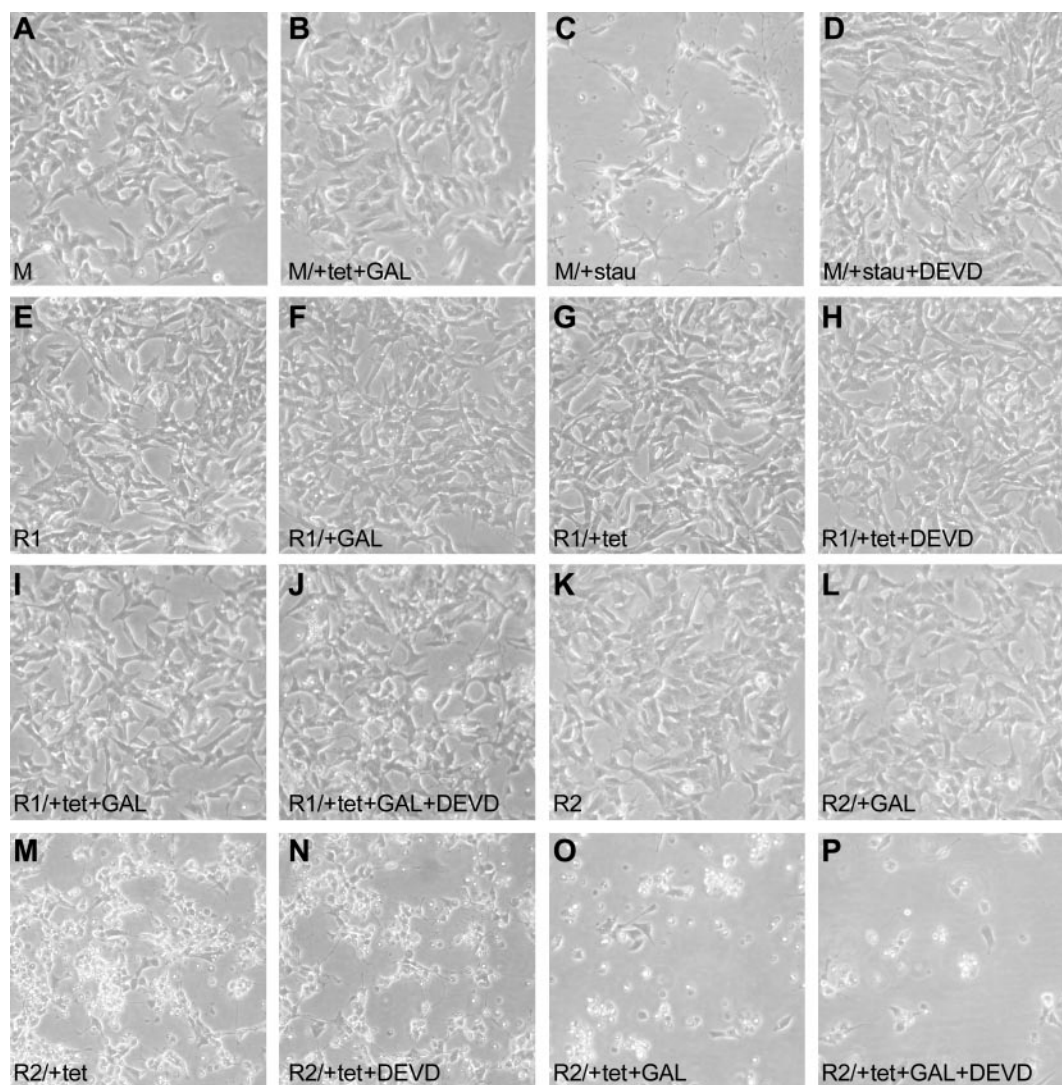


FIG. 3. Morphology of stable transfected SY5Y/mock (A–D), SY5Y/GalR1 (E–J), and SY5Y/GalR2 (K–P) cells treated for 48 h as indicated with 1  $\mu$ g/ml tetracycline (tet), 100 nM GAL, 1  $\mu$ M staurosporine (stau), or 50  $\mu$ M DEVD. Magnification of A–P,  $\times$ 80.

rosproprine-induced morphological changes in SY5Y/mock cells were inhibited by pretreatment of cells with 50  $\mu$ M DEVD (Fig. 3, C and D).

#### *Inhibition of cell growth and viability*

To study the effect of GAL receptors on the viability and proliferation of SY5Y/GalR1 and SY5Y/GalR2 cells compared with mock-transfected cells, cell viability assays were performed. We found that SY5Y/GalR1 as well as SY5Y/GalR2 cells showed negative proliferative activity in response to treatment with 1  $\mu$ g/ml tetracycline and 100 nM GAL for 72 h (Fig. 4), whereas SY5Y/mock cells did not show any changes. GAL significantly inhibited the proliferation of tetracycline-induced SY5Y/GalR1 cells by 19%. Because of endogenously secreted GAL by SH-SY5Y cells, treatment with tetracycline alone showed an inhibitory effect of 6% (Fig. 4A). GALP and AR-M1896 had no antiproliferative effect (Fig. 4B). Tetracycline induction alone already showed a pronounced effect on the viability of SY5Y/GalR2 cells with

a significant inhibition of cell proliferation by 60%, again due to endogenous expression and release of GAL in SH-SY5Y cells. Inhibition of cell proliferation was enhanced to 93% by the addition of 100 nM GAL (Fig. 4C).

DEVD was not able to block the inhibition of cell proliferation of tetracycline-induced SY5Y/GalR2 cells (Fig. 4C). The antiproliferative effect of GAL was 100-fold more potent on SY5Y/GalR2 cells than on SY5Y/GalR1 cells (Fig. 4, B and D). GALP and AR-M1896 were also potent agonists (Fig. 4D). GAL, GALP, and AR-M1896 did not exert antiproliferative effects on tetracycline-treated, mock-transfected cells.

#### *GalR2 receptor activation leads to apoptosis*

To investigate whether SY5Y/GalR2 cells die through apoptosis, cleavage of PARP, a major substrate for caspases, was investigated. Upon induction of apoptosis, the 113-kDa PARP protein is cleaved into 89- and 24-kDa fragments. Western blot analysis after tetracycline induction and GAL treatment for 48 h revealed that adherent SY5Y/GalR2 cells

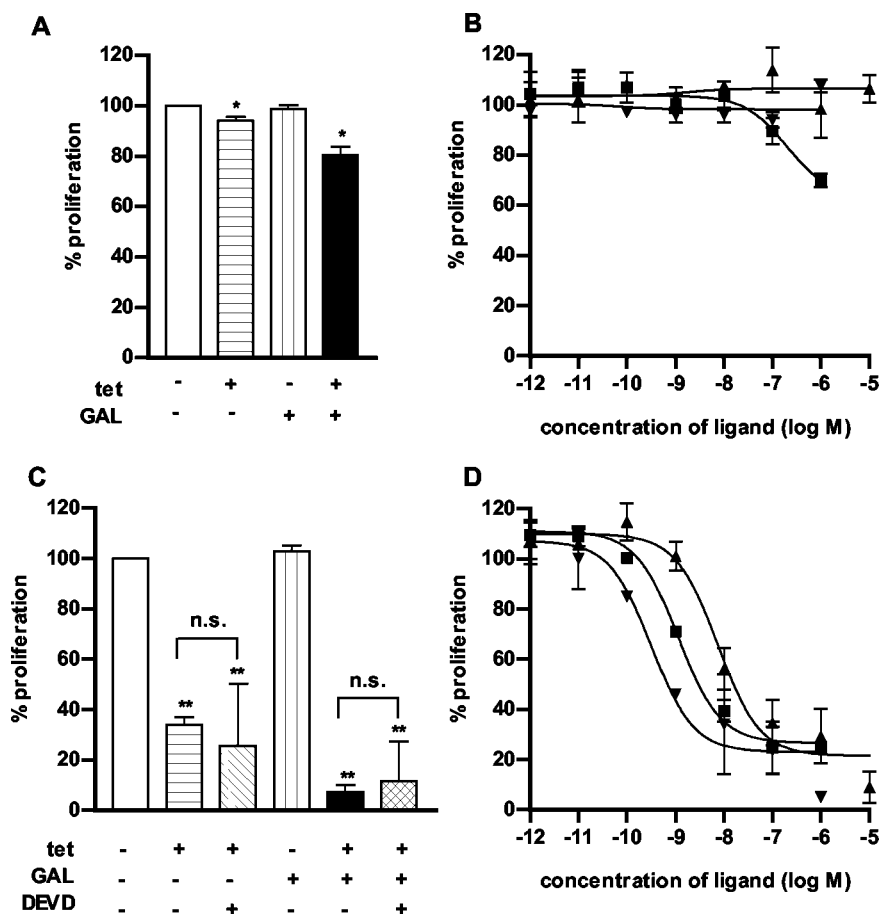


FIG. 4. Effect of GAL receptor activation on cell proliferation. A, SY5Y/GalR1 cells were treated for 72 h with 100 nM GAL in the absence or presence of 1  $\mu$ g/ml tetracycline (tet). Results are expressed as a percentage of the control values obtained with untreated cells, which were taken as 100%. Tetracycline decreased cell proliferation to  $95 \pm 5\%$ ; tetracycline and GAL decreased cell proliferation to  $81 \pm 10\%$ . GAL alone had no effect ( $99 \pm 4\%$ ;  $n = 10$ ). B, SY5Y/GalR1 cells treated with tetracycline and increasing concentrations of GAL showed a concentration-dependent inhibition of cell growth ( $EC_{50}$ ,  $190 \pm 83$  nM;  $n = 3$ ). C, Tetracycline decreased proliferation of SY5Y/GalR2 cells to  $40 \pm 3\%$ , tetracycline and GAL to  $7 \pm 3\%$ . GAL alone did not show any significant effect ( $105 \pm 6\%$ ;  $n = 14$ ). Pretreatment with 50  $\mu$ M DEVD (2 h) before induction with tetracycline did not significantly inhibit the decrease in cell proliferation ( $n = 4$ ). D, The inhibition of SY5Y/GalR2 cell growth was concentration dependent ( $EC_{50}$ : GAL,  $1.1 \pm 0.3$  nM; GALP,  $0.32 \pm 0.22$  nM; AR-M1896,  $7.4 \pm 2.9$  nM;  $n = 3$ ). All values are given as the mean  $\pm$  SE. \*,  $P \leq 0.01$ ; \*\*,  $P \leq 0.001$ . ■, GAL; ▼, GALP; ▲, AR-M1896.

contained only the 113-kDa protein, whereas detached SY5Y/GalR2 cells also displayed the cleaved 89-kDa protein (Fig. 5A). SY5Y/GalR1 as well as mock-transfected cells did not detach after 48 h with the same treatment, nor did they display the cleaved PARP protein (Fig. 5A).

Furthermore, a 3-fold increase in caspase-3 activity after tetracycline induction and GAL treatment for 48 h in SY5Y/GalR2 cells could be detected compared with untreated SY5Y/GalR2 cells (data not shown). The increase in caspase-3 activity after tetracycline and GAL treatment lies in the range of caspase-3 activity increase after treatment of SH-SY5Y cells with 3  $\mu$ M rotenone for 48 h, which also leads to apoptosis (23). SY5Y/GalR1 as well as mock-transfected cells did not show an increase in caspase-3 activity in response to the same treatment.

In addition, induction of SY5Y/GalR2 cells with 1  $\mu$ g/ml tetracycline and 100 nM GAL resulted in DNA laddering in detached cells (Fig. 5B), another feature of apoptotic, but not necrotic, cells. A time course of combined tetracycline/GAL treatment showed that the typical DNA ladders can be de-

tected after 24, 48, and 72 h of treatment, but not after 0, 6, and 12 h (data not shown). SY5Y/GalR1 and mock-transfected cells treated the same way did not show DNA ladders.

## Discussion

Here we show for the first time that induction of high levels of GalR2 expression mediates inhibition of cell proliferation and apoptosis in SH-SY5Y neuroblastoma cells. GAL has already been implicated in the regulation of cell proliferation and apoptosis. Recently, it was demonstrated that GAL increased the apoptotic index in immature rat thymocytes, indicating that GAL, probably acting through GalR1 and GalR3, exerts antiproliferative and proapoptotic effects on thymocytes (24). Another study showed that triple therapy with octreotide, GAL, and serotonin reduced tumor volume by increasing the number of apoptotic nuclei, resulting in regression of a rat colon carcinoma (25, 26). *In vivo* studies by Iishi *et al.* (27) revealed that prolonged administration of GAL significantly decreased the incidence of gas-

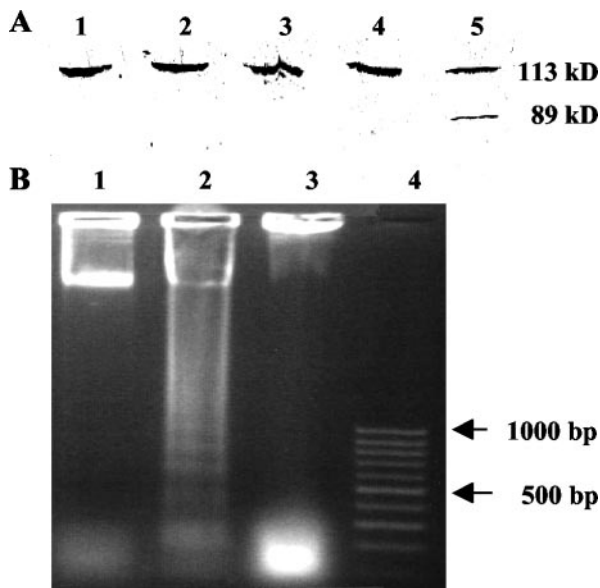


FIG. 5. Induction of apoptosis in SY5Y/GalR2 cells. Apoptotic cells could only be detected in the detached tetracycline/GAL-treated SY5Y/GalR2 cells by PARP Western blot (A) and DNA fragmentation analysis (B). Adherent SY5Y/GalR2 cells as well as SY5Y/GalR1 did not show apoptotic features in response to the same treatment. (A; lanes 1 and 2, SY5Y/GalR1; lane 1, without tetracycline induction; lane 2, with tetracycline and GAL treatment for 48 h; lanes 3–5, SY5Y/GalR2; lane 3, without tetracycline induction; lane 4, after treatment with tetracycline and GAL for 48 h; lane 5, detached cells collected after treatment with tetracycline and GAL for 48 h). B, SY5Y/GalR2 cells (lane 1, without tetracycline treatment; lane 2, detached cell fraction collected after 48 h of tetracycline and GAL treatment; lane 3, adherent cells collected after 48 h of tetracycline and GAL treatment; lane 4, 100-bp marker).

tric cancers in Wistar rats, indicating that GAL inhibits gastric carcinogenesis and that its effect may be related to the suppression of proliferation of antral epithelial cells. However, these GAL-mediated effects seem to be tumor and cell type specific, as GAL is also reported to act as a mitogenic agent in small cell lung cancer (28, 29) and on lactotroph proliferation (30). In addition, GAL appears to be a trophic factor in nerve regeneration (31, 32).

Our study includes the transfection of all three human GAL receptors cloned to date into the neuroblastoma cell line SH-SY5Y. However, only GalR1 and GalR2 turned out to be able to express high levels of GAL-binding sites and were functionally coupled, as determined by microphysiometry studies. The fact that the GAL binding levels of several clones of SY5Y/GalR3 were very low indicates that SH-SY5Y cells are not able to express GalR3 receptors in substantial amounts. There are several reasons that might account for SY5Y/GalR3 cells not being functional. The cells might lack the appropriate G protein(s) or components of signaling pathways that are necessary to mediate the GAL signal via GalR3. Also, the processing of the receptor into the cell membrane might be disturbed.

In contrast to SY5Y/GalR3 cells, tetracycline-induced SY5Y/GalR1 and SY5Y/GalR2 cells showed high GAL binding levels and also functional signaling, as determined by cytosensor studies. GAL application resulted in an increase in ECAR, reaching a plateau in SY5Y/GalR1 cells, which is

different compared with the biphasic response in the ECAR mediated via GalR1 previously reported in human Bowes melanoma cells (6).

As in tetracycline-induced SY5Y/GalR1 cells, similar patterns of an increase in ECAR after GAL application were seen in SY5Y/GalR2 cells. Although the acidification responses were of similar magnitude and kinetics in SY5Y/GalR1 cells, SY5Y/GalR2 cells exhibited desensitization upon repeated stimulation with GAL, suggesting the possibility of receptor internalization upon GAL binding in SY5Y/GalR2 cells. Whether the difference in receptor desensitization upon GAL stimulation between GalR1- and GalR2-transfected SH-SY5Y cells and/or the activation of differential signaling pathways via these receptors account for the observed major differences in their biological behavior remains unclear. This might also explain the differences detected in signaling of uninduced cells expressing low levels of GalR2 compared with induced SY5Y/GalR2 cells expressing high receptor levels. The difference in desensitization patterns in induced and uninduced SY5Y/GalR2 cells upon repeated stimulation with GAL remains unclear, but might be due to the amount of expressed receptor. The potency of GAL on changes in ECAR was 10-fold lower in uninduced SY5Y/GalR2 cells than in tetracycline-induced cells. Dependency of stimulation of signaling pathways on receptor density has also been described for the human  $\alpha_{1a}$ -adrenoceptor and the LH receptor (33).

Because cytosensor studies revealed that only GalR1 and GalR2, but not GalR3, receptors are functionally coupled in SH-SY5Y neuroblastoma cells, proliferation and apoptosis assays were only performed with SY5Y/GalR1 and SY5Y/GalR2 cells. Interestingly, cell viability assays revealed that GAL exerts an antiproliferative activity via GalR1 and GalR2 in tetracycline-induced transfectants. The low potency of GAL in SY5Y/GalR1 cells to inhibit cell proliferation compared with SY5Y/GalR2 cells indicates that only GalR2 could be of physiological relevance in neuroblastoma. This is supported by the study by Perel *et al.* (13), who suggested that the expression of GalR2 is responsible for the presence of GAL-binding sites in neuroblastoma. The difference in the responses of the two cell lines cannot be explained by a difference in receptor expression levels, as SY5Y/GalR1 cells possess more GAL-binding sites than SY5Y/GalR2 cells.

In addition to the antiproliferative effects, a proapoptotic effect of GAL receptor activation via GalR2 could be observed, as indicated by the degradation of PARP, the activation of caspase-3, and DNA fragmentation in SY5Y/GalR2 cells. Unexpectedly, we found that the caspase-3 inhibitor DEVD was ineffective in blocking apoptotic morphology and cell viability in GAL-treated, tetracycline-induced, SY5Y/GalR2 cells at a dose that prevented apoptotic morphology of staurosporine-treated SH-SY5Y cells. Therefore, caspase-3 is not an obligatory mediator of apoptosis triggered by activation of GalR2s in SY5Y cells. Similar mechanisms have been described for rotenone-induced apoptosis of SH-SY5Y cells (34).

Endogenous expression and secretion of GAL in SH-SY5Y cells are most likely the reasons why induction of SY5Y/GalR2 cells with tetracycline alone leads to substantial decreased cell proliferation and apoptosis. We know from this

and previous studies that the production of GAL in neuroblastomas is high enough to allow autocrine/paracrine activation of GAL receptors (12). The lack of GAL receptors in undifferentiated high stage neuroblastic tumors might be caused by the death of the cells expressing these receptors due to induction of apoptosis through GAL secreted by neuroblastoma cells. Therefore, we speculate that the lack of GAL receptors in neuroblastomas might represent a selective advantage, promoting rapid tumor growth. Expression of GAL receptors could be partially responsible for the occurrence of apoptosis in differentiated or regressing neuroblastic tumors (35).

The controlled expression of GalR2 receptors in neuroblastoma xenografts will elucidate whether GAL has an antiproliferative role in neuroblastoma development *in vivo* and whether GAL receptors could serve as therapeutic targets.

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